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HIGH DIVERSITY OF TRYPANOSOMA CRUZI DISCRETE TYPING UNITS CIRCULATING IN TRIATOMA IN WESTERN MEXICO

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HIGH DIVERSITY OF *TRYPANOSOMA CRUZI* DISCRETE TYPING UNITS CIRCULATING IN TRIATOMA IN WESTERN MEXICO

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by

Uyen Tran Thuong Nguyen

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Graduate Studies

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ABSTRACT

HIGH DIVERSITY OF *TRYPANOSOMA CRUZI* DISCRETE TYPING UNITS CIRCULATING IN TRIATOMA IN WESTERN MEXICO

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Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi*, which is transmitted to domestic and sylvatic mammals via the feces of hematophagous hemiptera of the subfamily Triatominae (Reduviidae). *Trypanosoma cruzi* is found only in the Americas and displays remarkable genetic diversity. Seven discrete typing units (DTUs) are currently recognized (TcI–TcVI and TcBat). In Jalisco, Mexico, where Chagas disease has a high prevalence rate, TcI has historically been the only DTU reported. This study focused on the molecular identification of *T. cruzi* DTUs circulating in *Triatoma* near the Estación de Biología Chamela, on the southwest coast of Jalisco, Mexico. I collected DNA from 95 *Triatoma* bugs. *Trypanosoma cruzi* infection was detected using PCR primers specific for the minicircle variable region of the parasite's kinetoplast DNA (kDNA). *Trypanosoma cruzi* DTUs were identified by amplifying the intergenic region of the mini-exon, and the genes *24Sα, 18S, TcSC5D*, and *TcMK*. Two species of *Triatoma* were collected, *Triatoma longipennis* and *T. bolivari*, with an overall infection rate of 59%. There was high genetic diversity of *T. cruzi* in my samples, with the DTUs TcI, TcII, TcIV, TcVI, and Tcbat being identified. This is the first report of TcVI and Tcbat in North America. In the *Triatoma* found to be infected, 96% had TcI, 35% TcII, 2% TcIV,

25% TcVI, and 2% Tcbat. Several vertebrate hosts for *Triatoma* were also identified from visible blood within Triatominaes' gut using PCR primers for *cytochrome b* and *cytochrome oxidase subunit I* genes. My observations indicate a much higher diversity of *T. cruzi* DTUs in *Triatoma* than previously reported in Jalisco. The results have important implications for understanding the geographical distribution of *T. cruzi* DTUs and epidemiology of Chagas disease in Mexico.

Keywords: *Trypanosoma cruzi*, Genotyping, Discrete typing units (DTUs), *Triatoma longipennis, Triatoma bolivari*, Seasonally dry tropical forest, Jalisco, Mexico.

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CHAPTER I

INTRODUCTION

Chagas disease (American trypanosomiasis) is caused by infection with *Trypanosoma cruzi*, a parasitic protozoan found in mammals wherever Triatominae insect vectors are found, between approximately 40^{o} N and S of the equator in the Americas ("Who, how, what and where ?" 2010). Chagas disease is listed by WHO as one of the top 17 most neglected tropical diseases that is caused by bacterial and parasitic infection, alongside ascariasis, trichuriasis, hookworm infection, leishmanisasis, and human African trypanosomiasis (Molyneux et al. 2016). Chagas disease is responsible for disability and early death in approximately one-third of those infected (Carabarin-Lima et al. 2013). The WHO reports that approximately 5-6 million individuals are infected with Chagas disease, more than 25 million are at risk, and 10,000 die each year in endemic areas (WHO 2010; 2015). Chagas disease has been increasingly detected in the US, Canada, and many European and Asian countries due to human migration between Latin America and the rest of the world (Coura and Viñas 2010).

In Mexico, it is estimated that 1.1 to 2 million people are infected with Chagas disease, predominately through vector-borne exposure (Carabarin-Lima et al. 2013). *Trypanosoma cruzi* infection has also increasingly been transmitted via blood transfusion(Carabarin-Lima et al. 2013), although mandatory serological screening nationwide is reducing this risk (Sánchez-González et al. 2016). In addition, there is no consensus on diagnostic methods and trypanocidal treatment is not administered to chronic patients (Carabarin-Lima et al. 2013). The disease is also becoming more urbanized due to human migration from rural areas to Mexican cities, mostly in search of jobs (Guzmán-Bracho 2001). There are 18 regions in Mexico, all rural, that have

endemic *T. cruzi* transmission, including the states of Oaxaca, Jalisco, Yucatan, Chiapas, Veracruz, Puebla, Guerrero, Hidalgo, and Morelos (Carabarin-Lima et al. 2013).

Due to a high genetic diversity, *Trypanosoma cruzi* is classified into 7 strains, known as discrete typing units (DTUs), namely TcI, TcII , TcIII , TcIV , TcV , TcVI, and Tcbat (Zingales et al. 2012). The dominant strain of *T. cruzi* in Mexico is TcI (Bosseno et al. 2002; Brenière et al. 2007), but recent studies have indicated other DTUs (TcII, III, IV, V) circulating in Triatominaes in Veracruz and Michoacán, Mexico (Ramos-Ligonio et al. 2012; Ibáñez-Cervantes et al. 2013). One study also reported many patients in Mexico were seropositive for TCII, which includes DTUs TcII-VI (Risso et al. 2011). These studies suggest either that the diversity of *T. cruzi* strains in Mexico is growing, or increased sampling has led to a better understanding of the complexity of DTUs within Mexico.

Jalisco has the highest prevalence of *T. cruzi* infection in Mexico, as 12% of the population was found to be seropositive (Carabarin-Lima et al. 2013), and only TcI has been previously reported there (Bosseno et al. 2002; Brenière et al. 2007). However, Jalisco is adjacent to Michoacán, where multiple *T. cruzi* DTUs have been identified (Ibáñez-Cervantes et al. 2013). Given these findings, Jalisco could potentially have greater diversity of *T. cruzi* DTUs than previously reported.

My study explores the prevalence and diversity of potential *T. cruzi* DTUs within their sylvatic *Triatoma* insect vectors inhabiting a tropical dry forest reserve in coastal Jalisco, Mexico. My objectives were to (a) evaluate the prevalence of *T. cruzi* in the local Triatominae, (b) determine the *T. cruzi* DTUs and haplotypes present in the region, (c) identify Triatominae vectors encountered near Estación de Biología Chamela (EBCh) in Jalisco, Mexico, and (d) when possible, determine which vertebrates are serving as hosts for Triatominae.

CHAPTER II

LITERATURE REVIEW

Brief Background

Trypanosoma is a genus in the class Kinetoplastida, and a monophyletic group of flagellated protozoa that is exclusively parasitic. Several trypanosomes cause serious disease in humans, *Trypanosoma brucei gambiense* and *T.b. rhodesiense* cause African trypanosomiasis (sleeping sickness), while *Trypanosoma cruzi* causes American trypanosomiasis (Chagas disease). Chagas disease was discovered by a Brazilian scientist Carlos Chagas (1879-1934) in 1909. He found a flagellated parasite in the intestine of a blood-sucking (hematophagous) bug and he named it *T. cruzi* after his mentor Oswald Cruz, the founder of the Oswaldo Cruz Institute in Brazil (Junqueira et al. 2010). After his discovery of Chagas disease, significant progress has been made in many aspects of this disease, including increased understanding of its epidemiology, parasite genome, immunology, and host-parasite interactions (Junqueira et al. 2010). However, it is still a serious public health concern in Latin America and non-endemic areas due to human migration (Brener and Gazzinelli 1997; El-sayed et al. 2005).

Chagas disease is transmitted to humans and mammals by blood-sucking bugs, which belong to the subfamily Triatominae (Hemiptera: Reduviidae). The disease is found in wildlife anywhere the vectors are found, from Argentina up to approximately 40^{o} North latitude in the Americas (2010). These bugs are attracted to warmth and CO_2 exhaled from mammals; therefore they usually bite the victims near their mouth at night while sleeping. Because of this, they have been nicknamed kissing bugs. Other common names are conenose bug, assassin bug, and triatomine bug. There are more than 130 species of kissing bugs, but only a few are known vectors for Chagas disease (Lent and Wygodzinsky 1979). Charles Darwin, in *Voyage of the*

Beagle, described being bitten by a kissing bug during his field trips to the Argentine pampas (Rassi et al. 2010). He later suffered debilitating symptoms consistent with Chagas disease infection (Rassi et al. 2010). Interestingly, *T. cruzi* DNA has been found in 9000-year-old mummified human tissues in northern Chile; the disease is believed to have been in the New World for 7-10 million years, long before humans migrated into the Americas (Aufderheide et al. 2004; Steverding 2014).

Morphology and Life Cycle

Trypanosoma cruzi is a eukaryotic single-celled flagellate. Besides having a nucleus, it possesses a single large and highly modified mitochondrian containing a network of circular DNA (called kinetoplast DNA or kDNA) that contain many copies of the mitochondrial genome. *T. cruzi* transitions between three different forms (epimastigote, trypomastigote, and amastigote) in its life cycle when it travels from its vector (kissing bugs) to its mammalian host (Fig. 1). The epimastigote and trypomastigote have a single flagellum that enables movement and attachment to the surface of the host cell (De Souza 1999). Epimastigotes are found in the intestinal tract of kissing bugs, are about 20 microns in length, and divide via binary fission. Its kDNA is positioned in the middle of the body, adjacent to the base of the flagellum posteriorly, and to nucleus anteriorly. Trypomastigotes are found in the blood stream of infected hosts. They are 15-25 microns in length and do not divide. Its kDNA is found at the most posterior portion of its body, adjacent anteriorly to the base of flagellum. The nucleus is found in the middle of the body. It has a characteristic C-shape if stained with Giemsa stains. Amastigotes live intracellularly, lack a flagellum and are among the smallest known eukaryotic cells (2.5–5 μm wide). Amastigotes are found within the cytoplasm of nucleated cells in their mammalian hosts. This stage is oval and is the dividing form within mammals.

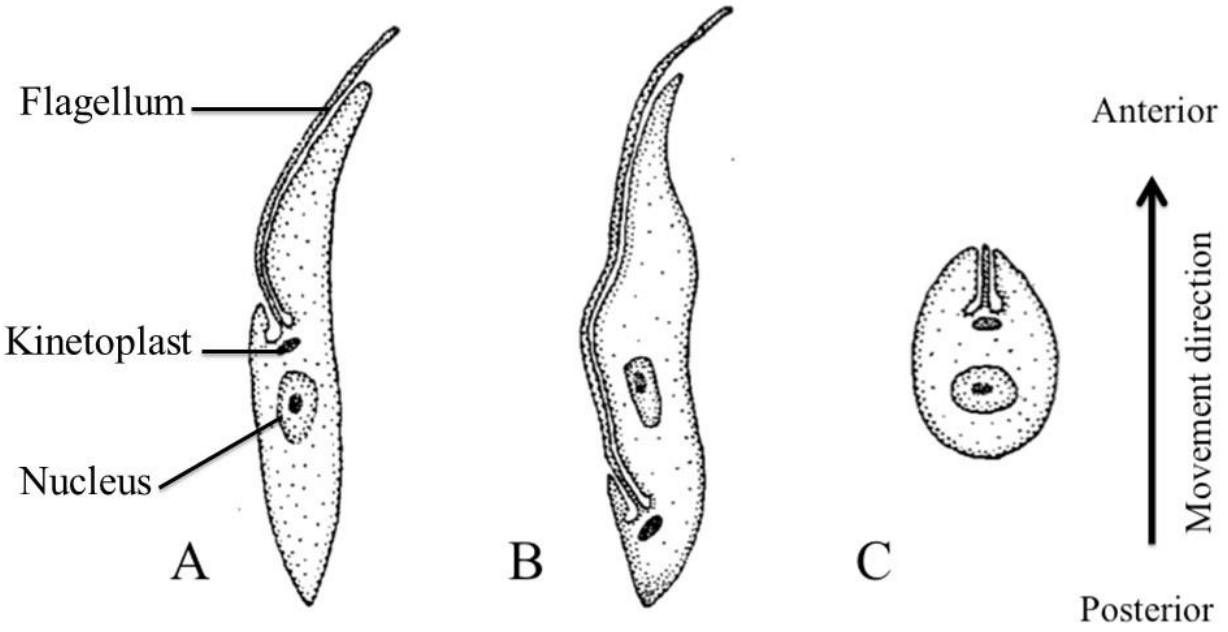

Fig. 1 Schematic illustration of *Trypanosoma cruzi* stages. Morphological differences included the size of each stage and the location of kDNA, nucleus, and flagellum. (A) Epimastigote, (B) Trypomastigote, (C) Amastigote (Schuster and Sullivan 2002).

The life cycle of *T. cruzi* starts when a kissing bug takes a blood meal from an infected mammalian host (Fig. 2). Blood containing trypomastigotes is ingested by the insect vector. The trypomastigotes transform into epimastigotes in the digestive tract of the bug. Epimastigotes differentiate into metacylic trypomastigotes in the feces of the kissing bug. During the blood meal, the infected bug defecates on the host, releasing highly infective parasites onto the source of the bloodmeal. The parasites are not passed by the bite itself. Metacylic trypomastigotes in the defecant enter the mammalian host's body through the bite wound, broken skin, or intact mucosal membranes. Inside the host, trypomastigotes enter the host nucleated cells and transform into a dividing form, amastigotes. Amastigotes mutiply inside the host cell until it ruptures, allowing them to invade other healthy cells.

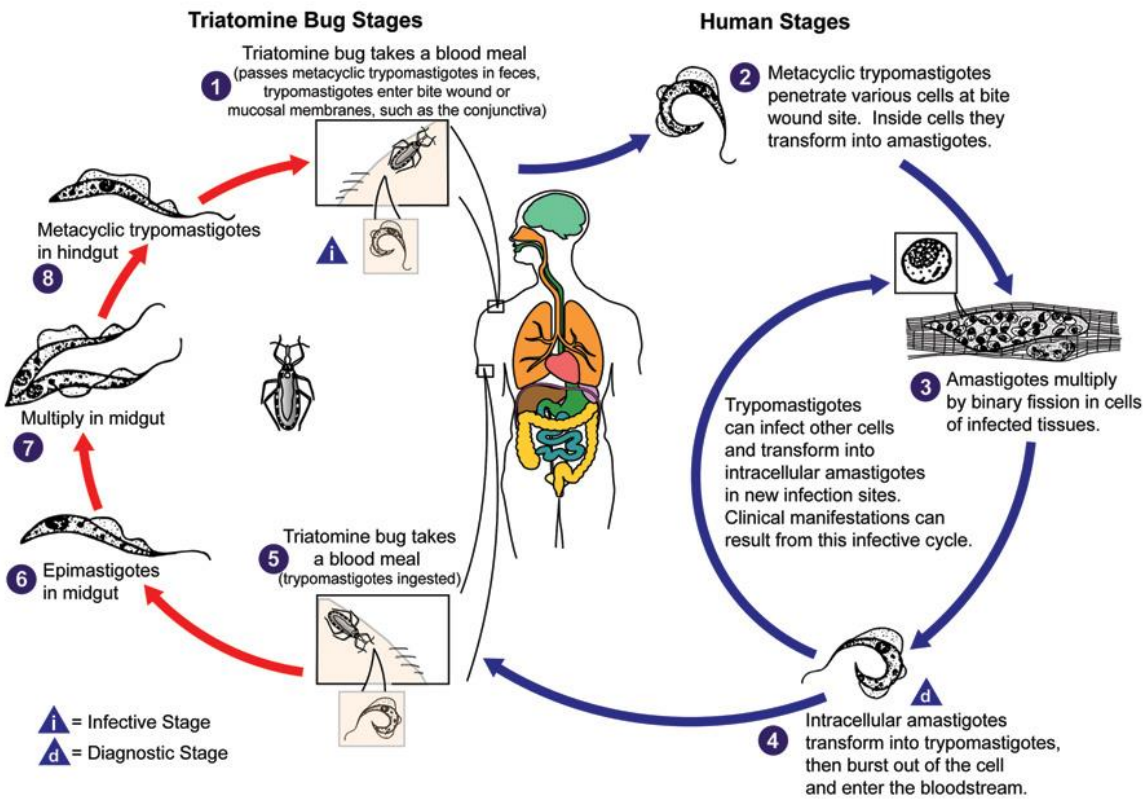

Fig. 2 Life cycle of *Trypanosoma cruzi* (Centers for Disease Control and Prevention 2015).

Chagas Disease

Acute phase

There are three phases of Chagas infection: acute, indeterminate, and chronic phases. The acute phase occurs after *T. cruzi* enters the victim's body and lasts for 4-8 weeks (Rassi et al. 2010). Most cases will pass unnoticed because the clinical symptoms are nonspecific and typical for many infections. Victims have flu-like symptoms, such as fever, nausea, vomiting, and diarrhea. An inflammatory lesion at the *T. cruzi* entry site (chagoma) and enlarged lymph nodes, liver, and/or spleen may be observed in the acute phase. If the entry point is the eye, it may cause swelling of the eye (Romana's sign) and conjunctivitis. Circulating trypomastigotes in blood are easily found during the acute phase. Acute infection can be cured with antiparasitic drugs such as benznidazole and nifurtimox (Rassi et al. 2010). Death may occur (<5-10%) in acute infected

individuals, often in children (4 years old or younger), from severe myocarditis and/or meningoencephalitis (Rassi et al. 2010).

When Chagas disease is transmitted congenitally by an infected mother during pregnancy, infected new born babies may develop acute symptoms at birth or a few weeks after birth, including hypotonicity, fever, hepatosplenomegaly, anemia, prematurity, low birth weight, and low Apgar score (Rassi et al. 2010). Miscarriage and placentitis can also occur during pregnancy due to maternal infection (Rassi et al. 2010).

Intermediate and chronic phases

After 8-12 weeks of the initial infection, the parasitemia becomes undetectable by microscopy and, without appropriate treatment, infected individuals will enter the intermediate form of Chagas disease (Bern et al. 2011). Patients may have a positive serological test, but never develop any clinical manifestations, and the parasite will be maintained for life (Machado et al. 2012). In some cases, individuals might not be aware of their infection but remain a potential source of *T. cruzi* transmission to vectors and humans (Bern et al. 2011).

Approximately, 30%-40% of infected individuals will develop the chronic phase of Chagas disease, which occurs 10-30 years after the initial infection (Rassi et al. 2010). Pathology of chronic Chagas disease, involved in heart, esophagus and colon, is classified into three clinical manifestations: cardiac, digestive and cardiodigestive. Digestive form of chronic Chagas disease is often observed in southern cone of South America and is rarely found in northern South America and Central America (Bern et al. 2011). Geographic distribution of disease pathology may be associated with different strains of *T. cruzi.* However, it has not been established which strain leads to which disease pathology of Chagas disease. Physiological factors of hosts, such as

genetic background, mitochondrial dysfunction, and immune competence, may be involved in the outcomes of clinical manifestation (Machado et al. 2012).

The digestive form occurs in 10-15% of those with chronic Chagas disease and is mainly found in the southern cone of South America: Argentina, Brazil, Chile, and Bolivia (Rassi et al. 2010). In the digestive form, *T. cruzi* injures the enteric nervous system, which regulates the function of gastrointestinal (GI) tract, resulting in dilation of organs of the GI tract (Machado et al. 2012). Although the enlargement of esophagus (megaoesophagus) and colon (megacolon) are the most common manifestations, enlargement of stomach, duodenum, jejunum, gallbladder, and choledochus are observed in some cases (Machado et al. 2012). Megaoesophagus causes difficulty in swallowing, regurgitation, excess salivation and malnutrition (Rassi et al. 2010). Megacolon, often occurring at the sigmoid segment, rectum, and descending colon, causes prolonged constipation, abdominal distension, and bowel obstruction (Rassi et al. 2010). Patients with megaoesophagus are more likely to develop esophagus cancer. However, colon cancer is not associated in patients with megacolon (Rassi et al. 2010). These clinical manifestations can ultimately lead to death in severe cases (Rassi et al. 2010).

The cardiac form occurs in 20-30% of individuals with chronic phase and is the most serious manifestation of chronic Chagas disease. Pathogenesis is not completely understood but believed to be due to parasite persistence (Bern 2015). Cardiomyopathy, the main manifestation of cardiac form, is characterized by a chronic inflammatory process of the heart, including all chambers and the conduction system (Bern et al. 2011). To infect the heart, trypomastigotes must pass through layers of extracellular matrix (ECM) to reach cardiac myocytes. Invasion by *T. cruzi* results in myocardial damage, such as ischemia, inflammation, oxidative stress, and necrosis, which leads to ECM degradation (Machado et al. 2012). The earliest signs of affected

patient are often conduction-system abnormalities and multiform premature ventricular contraction (Bern 2015). Patients also display symptoms of cardiomyopathy, including sinus and junctional bradycardias, atrial fibrillation, atrioventricular blocks, and non-sustained or sustained ventricular tachycardia (Bern 2015). As the disease progresses, dilated cardiomyopathy and congestive heart failure might occur. These symptoms lead to palpitation, presyncope, and risk of sudden death. Apical aneurysm is a the hallmark of chronic Chagas disease and is believed to be a result of ECM degradation (Machado et al. 2012). In addition, strokes and thromboembolic events may occur as a result of the dilated left ventricle or aneurysm (Bern et al. 2011). Sudden death is typical in advanced cardiomyopathy and accounts for more than half of deaths (55-65%), followed by heart failure, and thromboembolism (Rassi Jr et al. 2001). Thromboembolism can cause pulmonary and arterial embolization as well as stroke. Unexpected sudden death can also occur in patients with the indeterminate form of chronic Chagas disease, who do not have knowledge of their infection (Rassi Jr et al. 2001).

Innate and Adaptive Immune Responses During *T. cruzi* Infection

Innate and adaptive immune responses are two important parts of the human immune system. As its name suggests, the innate immune response consists of cells and proteins that humans are born with. It is responsible for non-specific defense mechanisms that will protect the body immediately against pathogens. The adaptive immune response is an antigen-specific mechanism and is initiated when the innate immune response fails to fight off the pathogen. Once a specific pathogen (antigen) is recognized and processed, the body will build up an army of specialized immune cells to overcome that specific pathogen. During the course of *T. cruzi* infection, the innate and adaptive immune systems play an important role for host survival and control of the parasite load.

Initial interaction of *T. cruzi* and host cells

Metacyclic trypomastigotes have the ability to invade any nucleated host cells. Once they penetrate the human body through broken skin or mucosal membranes, they encounter host tissue cells and immune cells, but have a preference to invade non-phagocytic cells rather than immune cells (Padilla et al. 2009). This allows the trypomastigotes to avoid the early immune response present in the blood, allowing the persistence of acute Chagas infection (Padilla et al. 2009). The parasite gains entry to the host cells in two ways: lysosome-dependent route or invagination of plasma membrane and fusion with lysosomes (Tardieux et al. 1992; Rodríguez et al. 1995; Rodríguez et al. 1996; Woolsey et al. 2003; Andrade and Andrews 2004). In both cases, it is confined to the host cell lysosome. A parasite-containing lysosome is called a parasitophorous vacuole. Lysosome fusion is necessary for a successful infection and trypomatigote-amastigote transformation (Woolsey et al. 2003; Andrade and Andrews 2004). The low pH environment of parasitophorous vacuoles activates a surface protein of the parasite called *trans*-sialidase. In the active form, *trans*-sialidase removes sialic acid, which is a protective coating on the inner layer of the parasitophorous vacuole and maintains the acidic environment. The absence of sialic acid ruptures the parasitophorous vacuole and releases trypomastigotes to the cytoplasm (Hall et al. 1992; Albertti et al. 2010). In the cytoplasm, the trypomastigotes transform into amastigotes. After several rounds of amastigote replication the infected cell bursts, releasing the parasites and allowing them to infect neighboring cells or travel in the blood stream to other parts of the body.

Pattern-recognition receptors

Pattern-recognition receptors (PRRs) are one of the first lines of host defense against *T. cruzi* and are expressed by cells of the innate immune response (Gazzinelli and Denkers 2006).

PRRs recognize the molecules of the pathogen, also called pathogen associated molecular patterns (PAMPs). Toll-like receptors (TLRs) are specialized types of the PRRs and are located at either the cell surface or within the endosome. They are mainly expressed by antigen-presenting cells, such as macrophage and dendritic cells. Binding of TLRs to PAMPs triggers the MyD88-dependent signaling cascade, which induces the activation of transcription factor NF-kB. Activated NF-kB diffuses into the cell nucleus and activates transcription of proinflammatory cytokines and chemokines, leading to the recruitment of phagocytic cells to the infected site as well as initiating the adaptive immune response. *T. cruzi* has several molecules that activate different TLRs, such as the mucin, glycoinositophospholipid (GIPL), and *T. cruzi* DNA and RNA. Mucin is a glycosylphosphatidylinositol (GPI)-anchored surface protein of *T. cruzi* (Almeida and Gazzinelli 2001). It binds and activates TLR2/TLR6, leading to the production of cytokines IL-12, TNF, and nitric oxide. GIPLs are free GPI anchors present in all forms of *T. cruzi*; they bind to and activate TLR4, leading to increased cytokine production. IL-12 also activates natural killer cells (NK). NK cells are cytotoxic and are able to kill parasite-infected cells. NK cells also activate macrophages by inducing production of the cytokine IFN-γ (Gazzinelli and Denkers 2006).

Unlike TLR2/TLR6 and TLR4, TLR7 and TLR9 are located on the intracellular membrane of endosomes and are activated by binding *T. cruzi* DNA and/or RNA molecules. Activation of TLR7 and TLR9 stimulates the production of Th1 proinflammatory cytokines (Cardoso et al. 2016). Nucleotide-binding oligomerization domain (Nod)-like receptors (NLR) are another type of PRRs and located in the cytoplasm of the cell. Studies show that NLRs are involved in controlling *T. cruzi* infection. However, the mechanism of NLRs is still unknown (Cardoso et al. 2016).

Complement system and *T. cruzi* infection

As the infected cell bursts, *T. cruzi* parasites enter the blood stream and encounter the complement system of the innate immune response. The complement system consists of three pathways: classical, alternative, and lectin. All pathways produce C3 convertase and C5 convertase, which lead to formation of the membrane attack complex (MAC). MAC forms pores in the lipid bilayer and lyses the parasites. The lectin pathway is activated by binding of mannan-binding lectin (MBL), H-ficolin, and L-ficolin to the surface of *T. cruzi*, and is responsible for approximately 70% of parasite lysis (Cestari et al. 2009; Cestari and Ramirez 2010; Cestari et al. 2013). The alternative pathway is activated by spontaneous cleavage of C3 to C3a and C3b (Cestari and Ramirez 2010). Anti-*T. cruzi* antibody is produced by immune cells and binds to the surface proteins of *T. cruzi*. This antibody then binds the C1 molecule and activates the classical pathway. To overcome defensive mechanisms of complement pathways, *T. cruzi* possess a group of molecules that prevent the activation of these pathways. For instance, calreticulin, a *T. cruzi* surface protein, interacts with several molecules of the lectin and classical pathways to prevent the formation of C3 and C5 convertases (Ferreira et al. 2004). *T. cruzi* complement regulatory protein (CRP), also known as GPI-anchored surface protein, binds to molecules involved in the initial steps of the classical and alternative pathways to render them inactive (Norris et al. 1991; Norris 1998). *T. cruzi* transmembrane protein, known as complement C2 receptor inhibition trispanning (CRIT), blocks the formation of C3 convertase in the lectin and classical pathways (Cestari et al. 2008; Cestari et al. 2009). In summary, *T cruzi* has diverse molecules that can disrupt the complement system, preventing the formation of C3 convertase, a key factor to parasite lysis. In addition, C3b molecule is produced by C3 convertase and is an opsonin, which enhances the phagocytosis of pathogens. Without C3 convertase, C3b molecule is not formed

and therefore prevents the phaygocytosis or lysis of *T. cruzi*. In addition, a study shows that *T. cruzi* triggers the release of host plasma membrane-derived vesicles, which inhibit the initial steps of classical and lectin pathways and facilitates the invasion of *T. cruzi* (Cestari et al. 2012).

Adaptive immunity against *T. cruzi* infection and immunodominance

Activation of TLRs not only activates the production of cytokines but also bridges the innate and adaptive immune responses. One of the hallmarks of an immunological response of adaptive immunity against *T. cruzi* infection is a strong and persistent T helper 1 (Th1) response as well as activating $CD8^+$ T cells. Th1 response is dependent on the TL7/TL9 activation (Junqueira et al. 2010). Activated Th1 cells produce IFN-γ, which activate macrophages and B cells, whose function is to produce specific antibodies for *T. cruzi* lysis and opsonization. *T. cruzi* also induces the production of IL-10, which triggers T helper 2 response. The cytokine IL-10 is known to inhibit the activation of Th1 immune response; therefore, activation of IL-10 is crucial for parasite survival (Geiger et al. 2016). In addition, the presence of *T.cruzi* causes intense polyclonal B cell activation, which is not specific for parasite antigens, and therefore may delay a parasite-specific humoral response (Cardoso et al. 2016).

In the infected cell, *T. cruzi* antigens are released in the cytosol, either from the parasite shedding or parasite lysis, will become available for processing and presentation by the major histocompatibility complex (MHC) class I. Although, $CD8^+$ T cells could potentially bind many *T. cruzi* antigens, they appear to preferentially recognize only a few *T. cruzi* epitopes, a process called immunodominance (Junqueira et al. 2010). Due to their abundance and high affinity to MHC and T cell receptor, the trans-sialidase and amastigote surface proteins are two important immunodominant antigens during *T. cruzi* infection (Junqueira et al. 2010; Cardoso et al. 2016). Studies suggest that immunodominance interferes with a broad recognition of *T.cruzi* antigens,

rendering the immune response unable to eradicate *T. cruzi* and leading to persistent infection and subsequent chronic Chagas disease (Rodrigues et al. 2009).

Epidemiology

There are two main transmission cycles of Chagas disease: wild (sylvatic) transmission cycle, occurring in wild kissing bugs and wild animals, and domestic transmission cycle, occurring in humans and household animals if kissing bugs live in human dwellings (home-dwelling kissing bugs). Peridomestic cycle is originated from both domestic and wild cycles, maintained by wild kissing bugs and domestic animals and occurs in areas surrounding human dwellings (Coura and Dias 2009). Chagas disease mainly occurs in developing countries with substandard rural housing and new urban or peri-urban areas, which allow infestation with the kissing bugs. Those infected often live in homes with mud walls and/or thatched roofing. If the kissing bugs inhabit the thatch roof, their feces may rain on the household items and food.

There are more than 180 wild mammalian species known to serve as *T. cruzi* reservoirs, including marsupials, xenarthra, bats, carnivores, lagomorphs, rodents, and non-human primates (Coura and Dias 2009). Domestic reservoirs are primarily dogs, cats, rats, mice, and cattle. Others animals, such as birds, reptiles, and fish could provide a blood meal for triatomine vectors. However, only mammals are known to support *T. cruzi* infection. The parasite's ability to survive in mammals is due in part to its expression of complement inhibitors that subvert innate immunity (Tambourgi et al. 1993; Norris 1998; Atayde et al. 2004; Cestari et al. 2008).

Vectors of *T. cruzi* are from the Reduviidae family (or subfamily Triatominae), mainly from the genera *Triatoma*, *Rohdnius* and *Panstrongylus*, with *T. infestans*, *T. dimidiata*, *R. proxilus*, and *P. megistus* being the most important vectors (Rassi et al. 2010; Carabarin-Lima et al. 2013). Most species are found from southern Argentina up to 40º North latitude in the United

States and from sea level up to 1500 meters of altitude (WHO 2002). Triatomines are nocturnal, have 5 nymphal stages, and adults of two sexes. They rely on blood exclusively for development and egg production. The probability of triatomines infected with *T. cruzi* is proportional to the number of blood meal taken. Therefore, older triatomines tend to have higher chances of having *T. cruzi* infection (Rassi et al. 2010). *T. infestans* is almost exclusively associated with human dwellings and is the main vector in the southern cone of South America, such as Uruguay, Chile, Brazil, and Argentina (WHO 2002; Vazquez-Prokopec et al. 2009; Rassi et al. 2010). *R. proxilus* is mainly found in Central America and northern South America (Rassi et al. 2010). Distribution of *T. dimidiata* is similar to *R. proxilus* but also extends to North America, including Mexico (Rassi et al. 2010). *P. megistus* is reported in South America and is the most important Chagas disease vector in Brazil (WHO 2002; Ribeiro et al. 2015).

Vector-borne transmission is the predominant route of infection with *T. cruzi*. However, organ transplantation, blood transfusion, ingestion of infected kissing bugs, *T. cruzi*–contaminated foods/liquids, and working in laboratories with live *T. cruzi*, are also potential routes of infection (Rassi et al. 2010). The infection can also occur congenitally via placenta and in ingestion of breast milk of an infected mother (Rassi et al. 2010; Steverding 2014). Furthermore, research demonstrating *T. cruzi* in gonads of mice suggests that Chagas disease could potentially be transmitted through sexual intercourse (Carvalho et al. 2009).

Even though Chagas disease is a major public health problem in endemic countries, little is known of the distribution of this disease in non-endemic areas. Chagas disease has been increasingly detected in the USA, Canada, and many European and Asian countries, due mainly to migration between Latin America and the rest of the world. Based on the immigration rate from Latin America to the USA and the prevalence of the disease, it is estimated that more than

300,000 individuals in the USA are infected with *T. cruzi* and more than half originated from Mexico. Since blood donation screening for *T. cruzi* started in 2009, 2,183 cases have been detected with *T. cruzi* infection in all states of the US except Wyoming and South Dakota (2016). In non-endemic areas, the main transmission mode is through transplantation, blood transfusion, and birth.

Diagnosis

Diagnosis of Chagas infection is accomplished with several different methods, which are used according to the phases of Chagas infection. During the acute phase, the parasitemia (circulating trypomastigotes in blood) is high and is easily detected in an anticoagulated blood or buffy coat by microscopic examination (Bern et al. 2007; Messenger et al. 2015). Trypomastigotes in blood are also visualized by staining with Giemsa or other stains and can be grown in specialized media in the laboratory (Bern 2015). Parasitemias decreases 2-3 months after infection, even without treatment, so microscopic examination is not accurate for patients with chronic infection. Since *T. cruzi* can be transmitted from chronically infected patients (blood transfusion, organ transplantation, and congenital infection) due to undetected infections, accurate diagnosis is critical (Bern 2015). Polymerase chain reaction (PCR) is a very sensitive diagnostic tool for the acute phase, congenital infection, and for recipients of an infected organ (Bern et al. 2011).

During the chronic phase, several serological tests are used to detect the presence of IgG antibodies against *T. cruzi* antigens, such as enzyme-linked immunosorbent assay (ELISA) or indirect and direct immunofluorescent antibody assays (IFA) (Bern 2015). Two serological tests are often required for a conclusive result. When results are different, a third test is used to confirm or disprove the diagnosis. Assays used as reference tests do not have high sensitivity and

accuracy for diagnosis, including radioimmunoprecipitation assay (RIPA) and trypomastigote excreted-secreted antigen immunoblot (TESA-blot) (Bern et al. 2011). Studies show that different *T. cruzi* strains possibly affect the sensitivity of serological tests (Bern et al. 2011).

Treatment and Control

There are two treatment options for Chagas disease: antitrypanosomal and symptomatic treatments (Bern et al. 2007). Antitrypanosomal treatment seeks to eradicate the parasites living in the patient's tissues. Benznidazole and nifurtimox are widely used as anti-parasitic drugs and are recommended for acute, congenital, and reactivated infections in individuals who have become immune-suppressed (Bern et al. 2011). These are offered to patients with chronic infections at the age of 18 or older (Bern et al. 2007). In the 1990s, studies found that benznidazole show 60% efficacy in chronic Chagas disease patients in treatment trials (de Andrade et al. 1996; Estani et al. 1998). Studies also show that benznidazole treatment prevents the progression of cardiomyopathy and decreases mortality in chronically infected adults (Viotti et al. 1994; Viotti et al. 2006).

Symptomatic treatment does not involve killing parasites, but lessening the cardiac and digestive manifestations that occur later in the chronic phase. For cardiac symptoms, single or several treatments may be used to prevent further complications, including medications, pacemaker, devices to regulate heart rhythm, or surgery (Rassi et al. 2010). Amiodarone has been widely used for patients with irregular heart rhythm and myocardial dysfunction. In severe cases, implantable cardioverter defilbrillators (ICDs) are also used. Patients with bradyarrhythmia (slow heart rate) are treated with medication and/or pacemaker. Treatment of gastrointestinal symptoms may include medication, diet, corticosteroids, or surgery (Rassi et al. 2010). Patients with megaoesophagus would have food and liquid deposited directly to the

stomach; in severe cases, esophagectomy is used. An early stage of colonic symptom can be treated with rich-fiber diet and abundant fluid intake, laxative, or enema. Patients with severe cases will undergo surgical organ resection.

A vaccine has not been developed for Chagas disease. The main focus of prevention is through vector control and prevention of non-vector borne transmission (Rassi et al. 2010). Application of insecticide is extensively used in triatomine-infested houses and endemic regions, as well as house improvement and health education. Screening of blood donors prevents *T. cruzi* transmission through organ transplantation and blood transfusion.

Genetic Diversity of *T. cruzi*

Trypanosoma cruzi is believed to have spread throughout the Americas by bats and is a clonal species, dividing by binary fission (Steverding 2014). This parasite was classified into two major lineages, *T. cruzi* I (TCI) and *T. cruzi* II (TCII). TCII has been further subdivided into five groups: IIa, IIb, IIc, IId, and IIe (Zingales et al. 2009). Recently, the lineages have been revised and classified into seven discrete typing units (DTUs), namely TcI (TCI), TcII (IIb), TcIII (IIc), TcIV (IIa), TcV (IId), TcVI (IIe), and the most recently proposed Tcbat (Marcili et al. 2009; Zingales et al. 2012). TcV and TcVI are hybrids of TcII and TcIII (Machado and Ayala 2001; Westenberger et al. 2005; De Freitas et al. 2006; Lewis et al. 2011). These hybrids may have arisen from two independent recombination events or a single incidence of hybridization followed by clonal divergence (Lewis et al. 2011). Tcbat, considered to be a bat-exclusive lineage of *T. cruzi*, has not been reported north of Panama (Breniere et al. 2016). Tcbatw is closely related to TcI and can be mistaken for TcI using standard molecular identification (Lima et al. 2015). It has been suggested that different DTUs cause different disease pathology of

Chagas disease, but it is unknown if there is an association between parasite DTUs and clinical manifestations (Rassi et al. 2010).

There are four proposed subgroups within the TcI DTU, known as haplotypes Ia, Ib, Ic, and Id (Herrera et al. 2009). Research suggests that haplotype Ia is associated with the domestic cycle and the vector *R. proxilus*, haplotype Ib is associated with humans, the peridomestic cycle, the vector *T. dimidiata*, haplotype Ic is associated with the peridomestic cycle, and haplotype Id is associated with the sylvatic transmission cycle (Herrera et al. 2009). A recent study reported that TcIa was has been found in three different states in Mexico, including in *T. dimidiata* in the Yucatan Penisula, in *T.* (*Meccus) picturatus* in Nayarit, and in an acute human case in Oaxaca (Monteón et al. 2014). Several studies have observed TcI circulating in vectors in Jalisco. However, haplotypes of TcI have not been previously identified (Magallón-Gastélum et al. 2004; Magallón-Gastélum et al. 2006; Brenière et al. 2007; Brenière et al. 2010)

Distribution of *T. cruzi* DTUs

The distribution of DTUs is distinct in North, Central, and South America (Fig. 3). TcI is the dominant strain (60% of the overall identifications) and frequently observed in sylvatic cycles but also in the domestic cycle throughout the Americas (Brenière et al. 2016). TcI and TcIV are the only DTUs reported in Central America (Higo et al. 2004; Ruíz-Sánchez et al. 2005; Iwagami et al. 2007; Brenière et al. 2016). In contrast, all DTUs have been detected in South America (Brenière et al. 2016). In the southern cone, TcII, TcV, and TcVI were proposed as the main agents of *T. cruzi* infection in domicilary transmission, whereas TcIII and TcIV were only occasionally found in humans (Brenière et al. 2016). In North America, all DTUs have been detected except TcVI and Tcbat (Brenière et al. 2016).

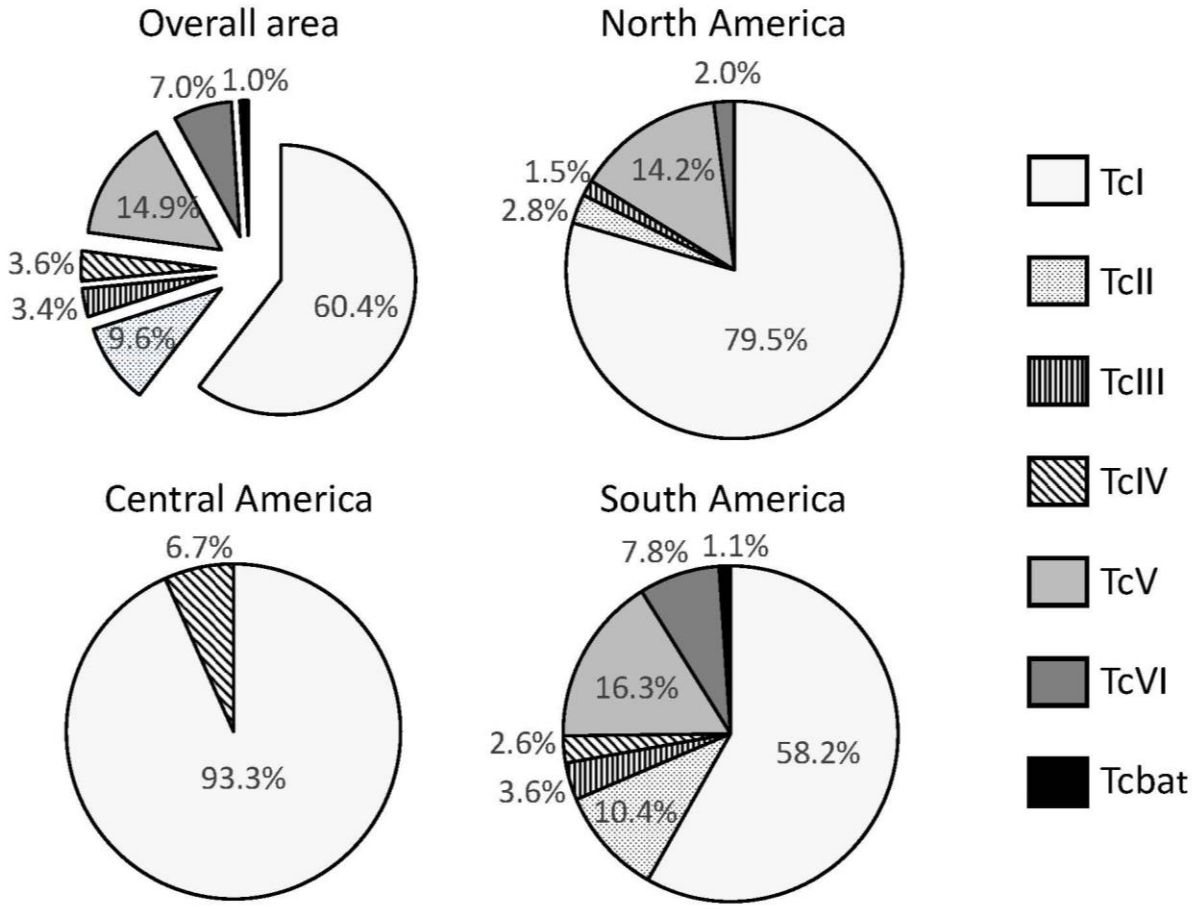

Fig. 3. Distribution of *T. cruzi* DTUs in North, Central, and South America (Brenière et al. 2016)

CHAPTER III

HIGH DIVERSITY OF *TRYPANOSOMA CRUZI* DISCRETE TYPING UNITS CIRCULATING IN *TRIATOMA* IN WESTERN MEXICO

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Abstract

Chagas disease is caused by the protozoan parasite *Trypanosoma cruzi*, which is transmitted to domestic and sylvatic mammals via the feces of hematophagous hemiptera of the subfamily Triatominae (Reduviidae). *Trypanosoma cruzi* is found only in the Americas and displays remarkable genetic diversity. Seven discrete typing units (DTUs) are currently recognized (TcI–TcVI and TcBat). In Jalisco, Mexico, where Chagas disease has a high prevalence rate, TcI has historically been the only DTU reported. This study focused on the molecular identification of *T. cruzi* DTUs circulating in *Triatoma* near the Estación de Biología Chamela, on the southwest coast of Jalisco, Mexico. We collected DNA from 95 *Triatoma* bugs. *Trypanosoma cruzi* infection was detected using PCR primers specific for the

minicircle variable region of the parasite's kinetoplast DNA (kDNA). *Trypanosoma cruzi* DTUs were identified by amplifying the intergenic region of the mini-exon, and the genes *24Sα, 18S, TcSC5D*, and *TcMK*. Two species of *Triatoma* were collected, *Triatoma longipennis* and *T. bolivari*, with an overall infection rate of 59%. There was high genetic diversity of *T. cruzi* in our samples, with the DTUs TcI, TcII, TcIV, TcVI, and Tcbat being identified. This is the first report of TcVI and Tcbat in North America. In the *Triatoma* found to be infected, 96% had TcI, 35% TcII, 2% TcIV, 25% TcVI, and 2% Tcbat. Several vertebrate hosts for *Triatoma* were also identified from visible blood within Triatominaes' gut using PCR primers for *cytochrome b* and *cytochrome oxidase subunit I* genes. Our observations indicate a much higher diversity of *T. cruzi* DTUs in *Triatoma* than previously reported in Jalisco. The results have important implications for understanding the geographical distribution of *T. cruzi* DTUs and epidemiology of Chagas disease in Mexico.

Keywords: *Trypanosoma cruzi*, Genotyping, Discrete typing units (DTUs), *Triatoma longipennis*, *Triatoma bolivari*, Seasonally dry tropical forest, Jalisco, Mexico

Introduction

Chagas disease (American trypanosomiasis) is caused by infection with *Trypanosoma cruzi*, a parasitic protozoan found in over 180 species of mammals wherever Triatominae insect vectors are found, between approximately 40^{o} N and S of the equator in the Americas ("Who, how, what and where ?" 2010). Chagas disease is responsible for disability and early death in approximately one-third of those infected (Carabarin-Lima et al. 2013). The WHO reports that approximately 5-6 million individuals are infected with Chagas disease, more than 25 million at risk, and 10,000 die each year in endemic areas (WHO 2010; 2015). Chagas disease has been increasingly detected in the US, Canada, and many European and Asian countries due to human migration between Latin America and the rest of the world (Coura and Viñas 2010). Although the vector does not live outside of the Americas, the risk of blood-borne transmission and burden of health care for those with chronic Chagas disease makes *T. cruzi* a global threat to public health.

In Mexico, it is estimated that 1.1 to 2 million people are infected with Chagas disease, predominately through vector-borne exposure (Carabarin-Lima et al. 2013). Human infections in Mexico come from both domestic and sylvatic transmission (Carabarin-Lima et al. 2013). *Trypanosoma cruzi* infection has also increasingly been transmitted via blood transfusion (Carabarin-Lima et al. 2013), although mandatory serological screening nationwide is reducing this risk (Sánchez-González et al. 2016). In addition, there is no consensus on diagnostic methods and trypanocidal treatment is not administered to chronic patients (Carabarin-Lima et al. 2013). The disease is also becoming more urbanized likely due to human migration from rural areas to Mexican cities, mostly in search of jobs (Carabarin-Lima et al. 2013). There are 18 regions in Mexico, all rural, that have endemic *T. cruzi* transmission, including the states of

Oaxaca, Jalisco, Yucatan, Chiapas, Veracruz, Puebla, Guerrero, Hidalgo, and Morelos (Carabarin-Lima et al. 2013).

The hematophagous triatomine vectors of *T. cruzi* include several species of the subfamily Triatominae (family Reduviidae) in 3 genera (*Triatoma, Rhodnius, Panstrongylus*). At least 31 species of *Triatoma* have been reported in Mexico with the most important vectors being *T. barberi, R. prolixus, T. dimidata, T. gerstaekeri, T. longipennis, T. mazzotti, T. mexicana, T. pallidipennis, T. phyllosoma, T. picturata,* and *T. rubida* (Ramsey et al. 2015). Control of Triatominae vectors in countries where domestic Chagas disease occurs primarily involves insecticide treatment of homes to reduce infestation. This has been shown to be the most cost effective option to control domestic Chagas disease (Coura and Dias 2009; Vazquez-Prokopec et al. 2009; Waleckx et al. 2014). In Mexico, where the majority of transmission is believed to be due to transient seasonal infestations from adult *Triatoma* dispersals from sylvatic habitats to human dwellings, the effectiveness of yearly treatment has not been studied (Waleckx et al. 2014). However, current control programs for mosquitoes to prevent Dengue transmission could potentially reduce *Triatoma* in residential areas (Carabarin-Lima et al. 2013).

Trypanosoma cruzi was originally assigned to two major lineages, *T. cruzi* I (TCI) and *T. cruzi* II (TCII). TCII was further subdivided into five groups: IIa, IIb, IIc, IId, and IIe (Zingales et al. 2009). Recently, the lineages have been revised and classified into seven discrete typing units (DTUs), namely TcI (TCI), TcII (IIb), TcIII (IIc), TcIV (IIa), TcV (IId), TcVI (IIe), and the most recently proposed Tcbat (Zingales et al. 2009). TcV and TcVI are hybrids of TcII and TcIII (Zingales et al. 2012). These hybrids may have arisen from two independent recombination events or a single incidence of hybridization followed by clonal divergence (Lewis et al. 2011). It has been suggested that different DTUs cause different disease pathology of Chagas disease,

but it is unknown if there is an association between parasite DTUs and clinical manifestations (Messenger et al. 2015).

Within TcI there is additional genetic diversity or haplotypes. The most commonly employed method for determining the haplotype of *T. cruzi* DTUs is based on genetic variability of the intergenic region of the mini-exon (Souto et al. 1996). By analyzing the intergenic region of the mini-exon, it is possible to determine four subgroups of TcI DTU, known as haplotypes Ia, Ib, Ic, and Id (Herrera et al. 2009). TcIa has been found in eastern Mexico but haplotype studies have not been undertaken in Jalisco (Monteón et al. 2014).

The dominant strain of *T. cruzi* in Mexico is TcI (Bosseno et al. 2002; Brenière et al. 2007), but recent studies have indicated other DTUs (TcII, III, IV, V) circulating in Triatominaes in Veracruz and Michoacán, Mexico (Ramos-Ligonio et al. 2012; Ibáñez-Cervantes et al. 2013). One study also reported many patients in Mexico were seropositive for TCII, which includes DTUs TcII-VI (Risso et al. 2011). These studies suggest either that the diversity of *T. cruzi* strains in Mexico is growing, or increased sampling has led to a better understanding of the complexity of DTUs within Mexico. Jalisco has the highest prevalence of *T. cruzi* infection in Mexico, as 12% of the population was found to be seropositive (Carabarin-Lima et al. 2013), and only TcI has been previously reported there (Bosseno et al. 2002; Brenière et al. 2007). However, Jalisco is adjacent to Michoacán, where multiple *T. cruzi* DTUs have been identified (Ibáñez-Cervantes et al. 2013). Given these findings, Jalisco could potentially have greater diversity of *T. cruzi* DTUs than previously reported. A recent survey showed that Jalisco is one of 10 Mexican states that have greatest risk for human exposure to infected Triatominae (Ramsey et al. 2015).

Our study explores the prevalence and diversity of potential *T. cruzi* DTUs within their sylvatic *Triatoma* insect vectors inhabiting a tropical dry forest reserve in coastal Jalisco, Mexico. Our objectives were to (a) evaluate the prevalence of *T. cruzi* in the local Triatominae, (b) determine the *T. cruzi* DTUs and haplotypes present in the region, (c) identify Triatominae vectors encountered near Estación de Biología Chamela (EBCh) in Jalisco, Mexico, and (d) when possible, determine which vertebrates are serving as hosts for Triatominae.

Materials and Methods

Study site

The Estación de Biología, Chamela (EBCh) is an ecological reserve and research station located in Jalisco in western Mexico (19º30'N, 105º03'W). The field station, owned and managed by the Universidad Nacional Autónoma de México (UNAM), is situated on 3,319 ha of undisturbed seasonally dry tropical forest (SDTF) and surrounding by an additional 13,142 ha of forest comprising the Chamela-Cuixmala Biosphere Reserve, one of few SDTFs protected areas on the planet (Ceballos 1995; Dirzo et al. 2011). The climate is highly seasonal, characterized by a pronounced dry season (Nov – Jun) and a 4-mo wet season (Jul – Oct) during which 80% of annual precipitation falls (Bullock 1986). From 1978 to 2015, mean annual precipitation was 829mm (range 300 – 1,300 mm; UNAM, EBCh meteorological data archives).

Collection and identification of triatomines

A total of 95 specimens were analyzed in this study. *Triatoma* were collected at three different periods during 2014- 2015 at EBCh. Eighteen *Triatoma* in our sample were captured in pitfall traps, initially installed in the Chamela forest for capturing lizards (García and Cabrera Reyes 2008), but which also proved to be useful in trapping *Triatoma* in this study. Several

Triatominaes were also collected by hand at EBCh due to their attraction to light (lit buildings and windows). All insects were euthanized by placing in -20ºC freezer for one hour.

Extraction of *Triatoma* DNA

Triatomines' hindgut contents were placed on FTA™ cards (GE Healthcare) to render any parasites non-infectious and to preserve DNA samples (Rogers and Burgoyne 1997; Kraus et al. 2011). Triatomines that were previously stored in 100% ethanol or dried were processed by macerating the abdomen in 500 µl distilled water followed by three cycles of freezing at -20ºC and thawing at room temperature before placing the mixture onto FTA™ card. For a 25 µl PCR reaction mixture, a 2 mm disc of the FTA card was cut out using Disposable Biopsy Punches, Integra™ Miltex® (Integra LifeScience). Prior to PCR, FTA discs were washed with FTA Purification Reagent (GE Healthcare) according to the manufacturer's instruction.

Diagnostic PCR

In this study, all amplification products were generated in a 25 µl reaction mixture containing a 2mm FTA disc, 0.2 µM of each primer, 12.5 µl of Apex Taq RED Master Mix (Genesee Scientific) and Nanopure™ Ultrapure H_2O, and generated in a C1000™ Thermal Cycler (Bio-Rad). All amplification products were separated on either TAE or TBE-agarose gel (Agarose GP2, Midsci™) with ethidium bromide and were visualized under UV light. Negative and positive controls (CL Brener, ATCC®) were carried out for all DNA samples.

Since Triatominae can be infected with either *T. cruzi* and/or *Trypanosoma rangeli*, all samples were subjected to PCR analysis using the primers TE and TR which are specific for *T. rangeli* (Fernandes et al. 2001) and amplify a 100 bp mini-exon PCR product (Table 1). Amplification products were separated on 2% TAE-agarose gel. To detect *T.cruzi* DNA, a diagnostic PCR was performed on all samples by amplifying the minicircle variable region of the

kinetoplast DNA (kDNA) using primers 121 and 122 (Table 1) (Cardoso et al. 1994). Samples that showed the diagnostic 330 bp fragment were considered *T. cruzi*-positive and were selected for molecular identification of *T. cruzi* DTUs.

Table 1 Primers, annealing temperature, and amplicon size used for identification of *T.cruzi* DTUs, triatomines, and blood meal source

Target organism	**Target gene**	**Primer name**	**5'-3' Primer sequence**	**Anneal ºC**	**Amplicon size**	**Ref.**
T. rangeli	Non-transcribed spacer region of mini-exon	TR TE	CCT ATT GTG ATC CCC ATC TTC G TAC CAA TAT AGT ACA GAA ACT G	50	100 bp	(Fernandes et al. 2001)
T. cruzi	Variable region of mini-exon	121 122	AAA TAA TGT ACG GGK GAG ATG CAT GA GGT TCG ATT GGG GTT GGT GTA ATA TA	57	330 bp	(Cardoso et al. 1994)
T. cruzi	Intergenic region of mini-exon	TC1 TC2 TC	GTG TCC GCC ACC TCC TTC GGG CC CCT GCA GGC ACA CGT GTG TGT G CCC CCC TCC CAG GCC ACA CTG	55	see table 2	(Souto et al. 1996)
T. cruzi	*TcSC5D*	TcSC5D-fwd TcSC5D-rev	GGA CGT GGC GTT TGA TTT AT TCC CAT CTT CTT CGT TGA CT	58	832 bp	(Cosentino and Agüero 2012)
T. cruzi	*TcMK*	Tc-Mev-kinase26-Fw Tc-Meb-kinase662-Rv	TTT TTG CAT GTC ATT TTG G AGC GGT CTT GTA ATG AGC AC	58	637 bp	(Cosentino and Agüero 2012)
T. cruzi	*24Sα*	D71 D72	AAG GTG CGC GAC AGT GTG G TTT TCA GAA TGG CCG AAC AGT	60	see table 2	(Souto and Zingales 1993)
T. cruzi	*18S*	V1 V2	CAA GCG GCT GGG TGG TTA TTC CA TTG AGG GAA GGC ATG ACA CAT GT	60	see table 2	(Clark and Pung 1994)
Vertebrate *Triatoma* sp.	mtDNA *cyt b*	Forward-cytb Reverse-cytb	GAG GMC AAA TAT CAT TCT GAG G TAG GGC VAG GAC TCC TCC TAG T	50	457 bp	(Mehus et al. 2013)
Vertebrate	mtDNA *COI*	Forward-COI_long Reverse-COI_long	AAC CAC AAA GAC ATT GGC AC AAG AAT CAG AAT ARG TGT T	50	663 bp	(Mehus et al. 2013)
Triatoma sp.	mtDNA *COI*	LCO1490 HCO2198	GGT CAA CAA ATC ATA AAG ATA TTG G TAA ACT TCA GGG TGA CCA AAA AAT CA	50	710 bp	(Folmer et al. 1994)

Molecular identification of *T. cruzi* DTUs

Trypanosoma cruzi genotyping was accomplished using two different methods. In the first method, we analyzed three gene fragments of *T. cruzi*: the intergenic region of the mini-

exon, and the genes *24Sα* and *18S*. Table 2 summarizes the expected size of amplification products. In the second method, we targeted the gene fragments of *TcSC5D* (putative lathosterol/episterol oxidase) and *TcMK* (mevalonate kinase) of *T. cruzi* (Cosentino and Agüero 2012).

Table 2 PCR amplification scheme and expected DNA fragments of different *T. cruzi* DTUs

	TcI	**TcII**	**TcIII**	**TcIV**	**TcV**	**TcVI**
Mini exon (TC, TC1 and TC2 primers)	350	300	-	-	300	300
***24Sα* rRNA (D71 and D72 primers)**	110	125	110	120,125,130*	100, 125	125
***18S* rRNA (V1 and V2 primers)**	175	165	165	155	155	-

*TcIV could have either 120, 125 or 130 bp (Brisse et al. 2001; Ramos-Ligonio et al. 2012)

*PCR amplification of the mini exon intergenic region***:** Multiplex PCR, using the primer set TC1, TC2 and TC, was used to amplify the mini-exon intergenic region (Souto et al. 1996). Amplification products were separated on 2% TAE-agarose gel. PCR amplification produced a 350bp fragment for TcI and/or 300bp for DTUs TcII, TcV, and TcVI.

*PCR amplification of the 24Sα***:** DTUs TcII, TcV, and TcVI were detected by amplification of 24Sα rRNA using primers D71 and D72 (Souto and Zingales 1993). Amplification products were separated by on 3% TAE-agarose gel due to small amplicon size. *24Sα* PCR amplified 125 bp fragment from TcII, 110 bp fragment from TcIII, 120, 125, and 130 bp fragments from TcIV, 110 and 125 bp fragments from TcV, and 125 bp fragment is produced from TcVI (Table 2).

*PCR amplification of the 18S***:** DTUs TcIII and TcIV both produce a 125 bp *24Sα* PCR product, therefore the DTUs were distinguished by amplification of the gene encoding *18S* rRNA using the primers V1 and V2 (Clark and Pung 1994). Amplification products were separated on 3% TAE-agarose gel. *18S* PCR amplified a 165 bp fragment from TcII, and no fragment is produced from TcVI (Table 2).

*DTU determination using the TcSC5D and TcMK genes***:** Cosentino and Agüero (2012) proposed a simple typing assay for determining *T. cruzi* DTUs that requires a single PCR amplification followed by either restriction enzyme digest or a direct sequencing assay. Amplification protocols for *TcSC5D* and *TcMK* were carried out according to the published protocol (Cosentino and Agüero 2012). *TcSC5D* gene fragments were used to determine DTUs TcI, TcII, TcIII, TcIV and Tcbat. *TcMK* fragments were used to determine TcV and TcVI. PCR was followed by enzyme digestion as described by Cosentino and Aguero (2012). Prior to enzyme digestion, PCR products were purified using DNA Clean & Concentrator-5 Kit (Genesee Scientific). A sample of 20 µl of purified *TcSC5D* PCR products were digested with 1 U of HpaI (NEB R0105) and 1 U SphI (NEB R0182) at 37ºC for 1 h. Digestions were performed in the CutSmart buffer (NEB B7204S). The digested fragments were resolved in 2% TBE-agarose gels. For *TcMK* gene, 20 µl of the purified *TcMK* PCR products were digested with 1 U of XhoI (NEB R0146) at 37ºC for 1 h. The digested fragments were resolved in 2.5% TBE-agarose gels.

Identification of Triatominaes

Identification of *Triatoma* found at EBCh involved using a dichotomous key (Lent and Wygodzinsky 1979), comparison to specimens deposited in the national collection of insects in the Institute of Biology, UNAM, and PCR analysis targeting a 457 bp region of mitochondrial *cytochrome b* (*cyt b*) and a 710 bp region of the *cytochrome oxidase subunit I* (*COI*) genes followed by sequencing (Table 1) (Folmer et al. 1994; Mehus et al. 2013). Two reference samples of *T. longipennis*, male and female, were sent to UNAM for identification due to difficulty with visual identification (Fig. 4).

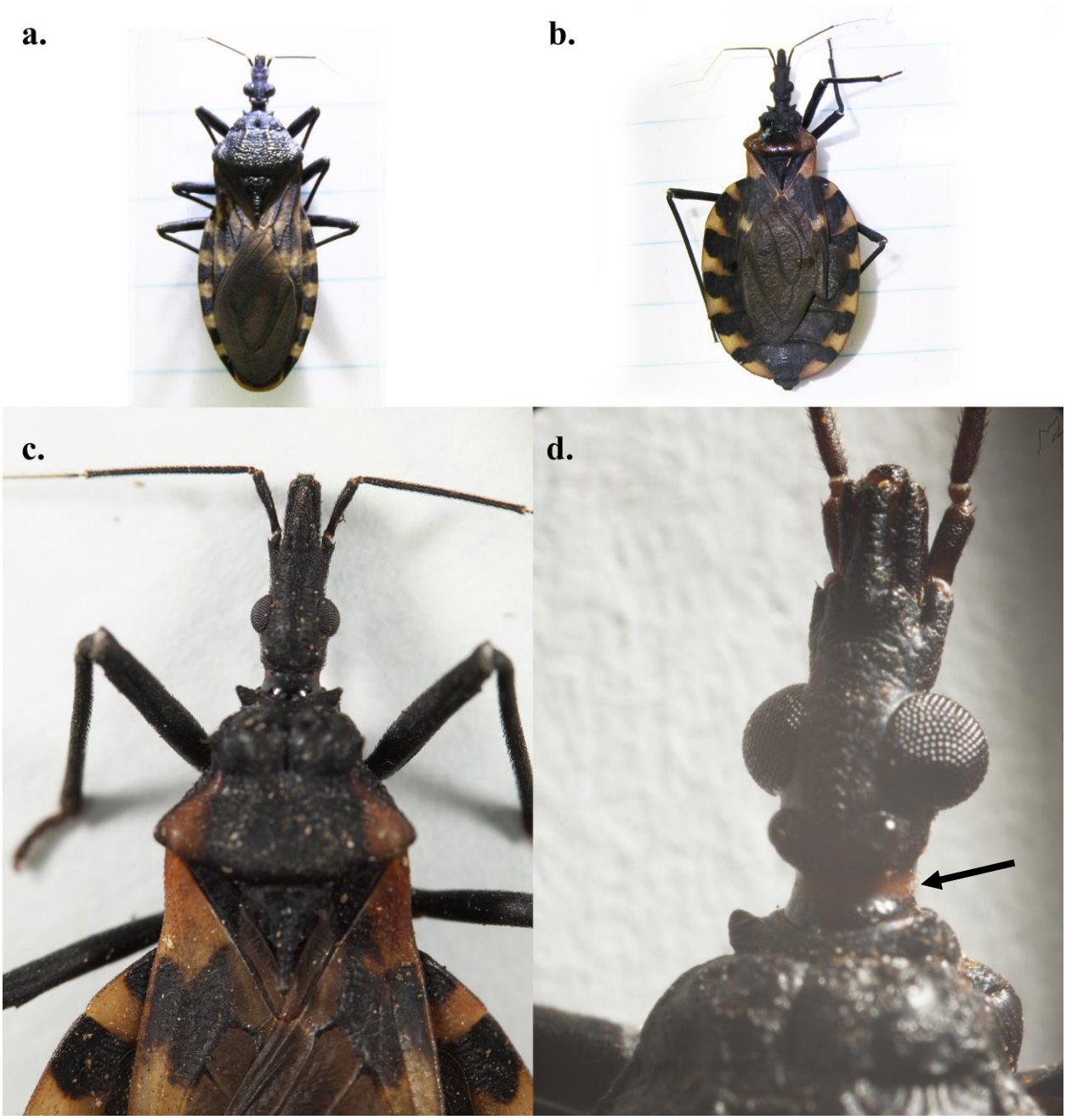

Fig. 4. *Triatoma* collected at *Estación de Biología, Chamela*. a. *Triatoma bolivari*, male, 24.4mm in length, collected on July 5, 2015 outside the library at EBCh, b. *Triatoma longipennis*, female, 35.5mm in length, collected on July 7, 2015 in a pitfall trap near Arroyo Colorado in the Chamela-Cuixmala Biosphere Reserve, c. close-up of *T. longipennis* head, corium, and pronotum, d. close-up of *T. longipennis* head and neck demonstrating yellow markings on side of neck (arrow)

The *COI* and *cyt b* genes of Triatominaes were PCR amplified, separated on 2% TAE-agarose gel, and the corresponding PCR products were purified and submitted for sequencing. The sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) at National Center for Biotechnology Information (NCBI, https://blast.ncbi.nlm.nih.gov/) to identify similarity to *Triatoma* sequences.

Identification of *Triatoma* blood meals

To identify the origin of *Triatoma* blood meals, 12 samples containing bllod were subjected to PCR analysis by targeting 457 bp region of *cyt b* genes, and a 663 bp region of the *COI* gene (Table 1) (Mehus et al. 2013). Amplification products were separated on 1.5% TAE-agarose gel, sequenced, and compared with deposited sequences in Genbank.

DNA Sequencing

The PCR products of the mini-exon intergenic region, *24Sα*, *18S*, *TcSC5D*, *TcMK*, *cyt b* and *COI* genes were excised and recovered from agarose using Zymoclean Gel DNA Recovery Kit (Genesee Scientific). The purified PCR products were sequenced by Genewiz, Inc (South Plainfield, NJ) and GenScript, Inc (Piscataway, NJ) using ABI 3730 DNA Analyzer. DNA sequences were manually aligned using BLAST, edited using BioEdit software and compared with the sequences in GenBank using BLAST search to identify the parasite DTUs, Triatoma and blood meals sources.

Results

***T. cruzi* detection in *Triatoma* specimens**

Ninety-five adult triatomines were collected at *EBCh* or nearby areas in Jalisco, Mexico. While collection efforts were attempted in the towns of Chamela and Emiliano Zapata no *Triatoma* were found. This may be due to recent spraying for Dengue in both villages, or the

non-domestication of local *Triatoma*. Two different species of *Triatoma* (Fig. 4) were found near the biological station, 61 *T. bolivari* (10 females and 51 males) and 34 *T. longipennis* (21 females and 13 males). A majority of the *T. bolivari* were found near light sources around the station, while the *T. longipennis* were predominately found in the forest.

The gut contents for all of the *Triatoma* collected were tested for the presence of conserved domains of mini-exon or kinetoplastid minicircle DNA using PCR primers specific for either *T. cruzi* or *T. rangeli*. *Trypanosoma rangeli* is non-pathogenic for mammals but causes pathology in Triatominae of the genus *Rhodnius*, and can be confused for *T. cruzi* when immunological test methods are used. *T. rangeli* are primarily seen in *R. prolixus* (Hoare 1972; Azambuja and Garcia 2005), although *T. rangeli* has been found in *Triatoma* in Colombia (Marinkelle 1968). None of the samples yielded *T. rangeli* amplicons of the non-transcribed spacer of the mini-exon gene using the primers TR/ME. The mini-exon intergenic region is a good target for PCR-based detection as there are over 200 tandem repeats each containing a highly conserved 39-nt exon sequence (Campbell et al. 2003). Of the 95 samples in our study, 56 (59%) were positive for *T. cruzi* using the 121/122 kinetoplast minicircle PCR primers. Because each parasite within an infected bug has thousands of 1.4 kb kinetoplast minicircles, the minicircles are an ideal target for *T. cruzi* detection (Thomas et al. 2007). Of the *T. bolivari* samples, 28 of 61(46%) were positive for *T. cruzi* and 28 of 34 *T. longipennis* samples (82%) tested positive for the parasite.

The various *T. cruzi* DTUs that we identified in our *Triatoma* samples are shown in Fig. 5. To identify *T. cruzi,* DTUs four different regions were targeted: 1) the mini-exon intergenic region, 2) the *24Sα* and *18S* ribosomal genes, 3) the *TcSC5D* gene, and 4) the *TcMK* gene. The limited amount of *T. cruzi* DNA that was preserved in samples on the FTA paper

made amplifying single copy genomic DNA difficult. Therefore, multiple methods were employed to differentiate DTUs from the 56 positive samples (Tables 3). Thirty of those samples revealed a mini-exon intergenic region PCR product of 350 bp and were thus identified as infected with only TcI (Table 4). One sample produced a PCR product of 300 bp that corresponded to either TcII, TcV, or TcVI. Further analysis using the *24Sα* and *18S* genes produced 125 and 165 bp products respectively identifying DTU TcII (1/56, 2%). One sample showed neither a 300 bp nor a 350 bp of the mini-exon gene suggesting TcIII or TcIV. This sample produced a 125 bp product of the *24Sα* gene and a 155bp product of the *18S* gene, identifying it as TcIV (1/56, 2%).

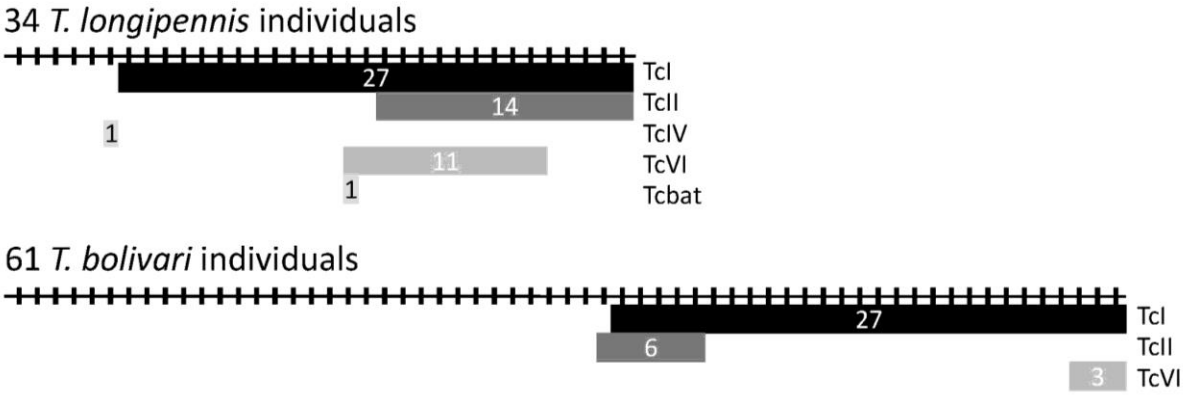

Fig. 5 The distribution of *T. cruzi* DTUs in EBCh *Triatoma***.** Each hatch mark represents an individual *Triatoma*, boxes below the line represent the different DTUs found in each individual. A. Out of 34 *T. longipennis*, 28 (82%) contained *T. cruzi* DTUs. B. Out of 61 *T. bolivari* 28 (46%) contained *T. cruzi* DTUs

Twenty-four of the 56 *T. cruzi* positive samples produced two products of 350 bp and 300 bp and therefore contained multiple DTUs. These samples with mixed infections of *T. cruzi* DTUs were further analyzed using *24Sα* and *18S* ribosomal genes; however, most of the PCRs produced amplification products of 110bp (*24Sα*) and 175bp (*18S*), which demonstrates only TcI (Table 2). The amount of TcI DNA might be more abundant in samples with mixed infections, or

the primers may preferentially amplify TcI over other DTUs, which would explain why the *24Sα* and *18S* primers tended to pick up only the ribosomal genes from TcI.

Of the 56 positive samples, 35 amplified with primers TcSC5D-fwd/TcSC5D-rev, which target the genomic single copy *TcSC5D* gene. We were not able to amplify the *TcSC5D* gene in the remaining 31 positive samples because not enough *T. cruzi* DNA was preserved in the FTA paper. Four different DTUs, TcI, TcIV, TcVI, and Tcbat, were found in the 35 samples using the *TcSC5D* gene, a restriction enzyme digestion assay, and DNA sequencing (Table 3 and Table 4). The DTUs TcV and TcVI are two independent hybrids that are derived from TcII and TcIII (Zingales et al. 2012). Therefore, discrimination of TcV and TcVI by sequencing *TcSC5D* amplification products does not identify the strain. By amplifying a different gene (*TcMK*) and using a restriction enzyme assay we were able to distinguish between TcV and TcVI.

Considering the data together, 54 of 56 (96%) samples were infected with TcI, 20/56 (36%) with TcII, 14/56 (25%) with TcVI, 1/56 (2%) with TcIV, and 1/56 (2%) with Tcbat (Fig. 5). Many of the infected *Triatoma* 24/56 (42%) contained mixed DTU populations.

DNA sequencing and data deposition

T. cruzi DTU identification was confirmed by sequencing of the mini-exon intergenic region, and the *TcSC5D* and *TcMK* genes (Table 5). We were able to confirm haplotype TcIa in all of the TcI positive samples. Sequence data also confirmed the PCR analysis for TcII, TcIV, TcVI, and Tcbat.

Triatoma bolivari found at EBCh were identified from physical characteristics and by sequencing the *cyt b* gene (Table 6). The *cyt b* gene of *T. bolivari* had a 91% identity with the only reported *cyt b T. bolivari* sequence, which was collected at EBCh. There was also a 100% identity with the *COI* gene of a *Triatoma* sp. deposited in the American Museum of Natural

History, but this specimen had no location or species information. For *T. longipennis*, the *COI* gene did not have high homology to any deposited sequences and the *cyt b* gene only had 87% identity with a *T. longipennis* sequence on GenBank (Table 6).

Table 3 Molecular identification of *T. cruzi* DTUs and DNA sequencing suggest mixed infections in triatomines

	No of samples	**Mini-exon**	***24S* and *18S***	**TcSC5D and TcMK[#]**	**Total DTUs**
Tl & *Tb*	11	TcI	TcI	TcI*	TcI
Tl & *Tb*	19	TcI	TcI	ND	(n=30)
T. bolivari	1	TcII	TcII	TcII**	TcII (n=1)
T. longipennis	1	ND	TcIV	TcIV*	TcIV (n=1)
T. bolivari	9	TcI, TcII	TcI	TcI*	TcI, TcII
Tl & *Tb*	1	TcI, TcII	ND	ND	(n=10)
Tl & *Tb*	3	TcI	TcI	TcI*, TcVI**	TcI, TcVI
T. bolivari	1	TcI	-	TcVI*	(n=3)
T. longipennis	2	TcI, TcII	TcI	TcI*, TcVI**	TcI, TcII, TcVI
	7	TcI, TcII	-	TcI**,TcVI*	(n=9)
T. longipennis	1	TcI	TcI	Tcbat*, TcI**, TcVI**	TcI, TcVI, Tcbat (n=1)

*Parasite DTUs were confirmed by both enzyme digestion assay and DNA sequencing results
**Parasite DTUs were confirmed by enzyme digestion assay only
[#] *TcMK* gene used for TcV and TcVI discrimination
Tl & Tb = T. longipennis and *T. bolivari*
ND = no data was obtained

Table 4 Frequency of *T. cruzi* DTUs and mixed infections in triatomines

Triatomines	**Single and mixed infection frequency**						
	TcI only	**TcII only**	**TcIV only**	**TcI, TcII**	**TcI, TcVI**	**TcI, TcII, TcVI**	**TcI, TcVI Tcbat**
T. longipennis (n=28)	11	-	1	5	1	9	1
T. bolivari (n=28)	19	1	-	5	3	-	-
Total (n=56)	30	1	1	10	4	9	1

Table 5 GenBank accession numbers of *TcSC5D*, *TcMK*, and Mini-exon sequences of *T. cruzi* DTUs

***T. cruzi* DTUs**	***TcSC5D***		***TcMK***		**Mini-exon**	
TcI #	KX858838*	CP015687 (99%) JN050565 (99%)	-	-	KX858836*	AM259467 (TcIa) (99%) AM259469 (TcIb) (97%) AM259474 (TcIc) (96%) AM259473 (TcId) (94%)
TcII	KX987101*	JN050574 (99%) JN050569 (99%)	-	-	KX858837*	KM376439 (99%) KM376435 (99%)
TcIV	KX858839*	JN050564 (99%) JN050568 (99%)	-	-	-	-
TcVI	KX858840*	XM_797152 (100%) JN050576 (93%)	KX874594*	KR350585 (99%) XM_797435 (99%)	-	-
Tcbat	KX858841*	TCC1122 (99%)	-		-	-

% identity to deposited sequences in GenBank is provided in parenthesis.
*Sequences were obtained in this study.
Haplotype TcIa was identified by DNA sequence of mini-exon.

Triatoma bolivari found at EBCh were identified from physical characteristics and by sequencing the *cyt b* gene (Table 6). The *cyt b* gene of *T. bolivari* had a 91% identity with the only reported *cyt b T. bolivari* sequence, which was collected at EBCh. There was also a 100% identity with the *COI* gene of a *Triatoma* sp. deposited in the American Museum of Natural History, but this specimen had no location or species information. For *T. longipennis*, the *COI* gene did not have high homology to any deposited sequences and the *cyt b* gene only had 87% identity with a *T. longipennis* sequence on GenBank (Table 6).

Table 6 GenBank accession numbers of *cyt b* and *COI* sequences of *Triatoma*.

***Triatoma* sp.**	***Cyt b***		***COI***	
Triatoma bolivari	KY193790*	JQ282718 (91% *T. bolivari*)	KY033219*	AY252963 (100% *Triatoma* sp.)
Triatoma longipennis	KY033227*	JN5858554 (91% *T. dimidiata*) DQ198815 (87% *T. longipennis*)	KY033220*	KC249392 (76% *T. vandae*)

% identity to deposited sequences in GenBank is provided in parenthesis.
*Sequences were obtained in this study.

Twelve *Triatoma* had visible blood within their gut contents. The blood meal source was identified by amplifying the *cyt b* and *COI* genes. The DNA sequences were queried for similar sequences using NCBI's nucleotide BLAST search. Table 7 contains the percent identity of the various blood meal sources and their accession numbers. Of the twelve Triatoma containing visible blood, we were able to obtain *cyt b* and *COI* sequences for eleven samples (Table 7). One sample had a low match (81%) for *cyt b*, but a 100% match for *COI* of *Gallus gallus*. One sample had 96% match for *cyt b* of *Sciurus aureogaster*, but we were not able to compared *COI* sequence of *S. aureogaster* to other sequences since it is not available on GenBank.

Table 7 Identification of the triatomines' blood meals using *cyt b* and *COI* genes.

***Triatoma* sp.**	**#**	***T. cruzi* DTUs**	**Blood meal sources**	**Accession #**			
				Cyt b		***COI***	
T. longipennis	2	TcIa	White-tailed deer (*Odocoileus virginianus*)	KX874595*	KM612275 (99%)	KY033224*	JN632673 (99%)
	1	TcIa, TcII					
	1	TcIa	Marble toad (*Incilius* (*Bufo) marmoreus*)	KX874599*	HM563957 (99%)	KY033223*	JN867979 (99%)
	1	TcIa, TcII	Dog (*Canis familiaris*)	KX874598*	KU291082 (99%)	KY033225*	KU696410 (100%)
	1	TcIa, TcII	Human (*Homo sapiens*) Dog (*Canis familiaris*)	KX874596* KX874598*	AP008916 (100%) KU291082 (99%)	KY033221* KY033225*	KT725864 (100%) KU696410 (100%)
T. bolivari	2	N/A	Mexican gray squirrel (*Sciurus aureogaster*)	KX874597*	KC737847 (96%)	KY033226*	-
	1	TcIa					
	1	TcIa, TcVI	Human (*Homo sapiens*)	KX874596*	AP008916 (100%)	KY033221*	KT725864 (100%)
	1	TcIa	Chicken (*Gallus gallus*)	KY084833*	EF076670 (81%)	KY033222*	KT780168 (100%)

% identity to deposited sequences in GenBank is provided in parenthesis.
*Sequences were obtained in this study.

Discussion

T. cruzi infection rate at EBCh

We found many *Triatoma* infected with *T. cruzi* within EBCh, with an overall infection rate of 59% (82% of *T. longipennis* and 46% of *T. bolivari)*. Previous studies reported the *T. cruzi* prevalence in *Triatoma* from Jalisco to range from 46-57%, a result similar to ours (Magallón-Gastélum et al. 2004; Brenière et al. 2007; Brenière et al. 2010). The very high

infection rate observed in *T. longipennis* (28/34 or 82%) may be an artifact of small sample size and further collection will provide a clearer estimate of the prevalence of *T. cruzi* in this Triatominae.

High diversity of *T. cruzi* DTU at EBCh

The diversity of *T. cruzi* DTUs has not been thoroughly investigated in Jalisco; only TcI has previously been reported (Bosseno et al. 2002; Brenière et al. 2004; Magallón-Gastélum et al. 2004; Magallón-Gastélum et al. 2006; Brenière et al. 2007; Walter et al. 2007; Brenière et al. 2010). Our findings indicate there is greater diversity of *T. cruzi* DTUs than previously reported. This is in agreement with studies from other states in Mexico that indicate high frequencies of non-TcI strains. TcIII, TcIV and TcV were observed in Veracruz (12-27% each) (Ramos-Ligonio et al. 2012) and TcII, TcIII, and TcIV were found in Michoacán (6-20% each) (Ibáñez-Cervantes et al. 2013).

In our study, TcI, and in particular the haplotype TcIa, was found in 96% of infected Triatominae. This result suggests that TcIa is the dominant strain circulating in mammals in coastal Jalisco. Several studies have observed TcI circulating in vectors in Jalisco; however, haplotypes of TcI have not been previously identified (Magallón-Gastélum et al. 2004; Magallón-Gastélum et al. 2006; Brenière et al. 2007; Brenière et al. 2010). Our study confirms the presence of the haplotype TcIa in Jalisco. A recent study reported that TcIa was also found in three different states in Mexico, including in *T. dimidiata* in the Yucatan Pennisula, in *T. (Meccus) picturatus* in Nayarit, and in an acute human case in Oaxaca (Monteón et al. 2014).

The distribution of DTUs is distinct in North, Central, and South America. TcI and TcIV are the only DTUs reported in Central America (Brenière et al. 2016). In contrast, all DTUs have been detected in South America. In the southern cone, TcII, TcV, and TcVI were proposed as the

main agents of *T. cruzi* infection in domicilary transmission, whereas TcIII and TcIV were only occasionally found in humans (2010; Brenière et al. 2016). TcI was frequently observed in sylvatic cycles but also in the domestic cycle throughout the Americas (Brenière et al. 2016). In North America, all DTUs have been detected, except TcVI and Tcbat (Brenière et al. 2016). However, our findings now confirm the presence of TcVI and Tcbat in North America.

The high diversity of DTUs in a small area could be attributed to human and animal movement. It is unknown if the high DTU diversity observed is novel to Mexico, or due to increased surveillance. If the non-TcI DTUs are due to migration, visiting scientists at EBCh may be contributing to the novel DTUs seen near the biological station. It would be interesting to see if sampling other locations in costal Jalisco would yield the same high diversity as in our study at EBCh. The high volume of traffic moving along Coastal Mexican Federal Highway 200 (Carretera Federal 200) through the biological reserve also contributes to the possibility that high diversity might be due to migration through the region, complicating understanding of which strains are historically found in Jalisco versus imported DTUs.

First report of DTUs TcVI or Tcbat in North America

To our knowledge, this is the first report of either TcVI or Tcbat being detected in North America (Brenière et al. 2016). TcVI was found in 25% (14/56) of the *Triatoma* with *T. cruzi*. Because of this relatively high prevalence, our finding suggests that TcVI is likely well established in the forest surrounding EBCh. TcVI has been reported throughout South America from Argentina to northern Colombia (Messenger et al. 2016). In the southern cone region, TcVI is associated with domestic transmission cycles where severe chronic Chagas disease and congenital transmission are prevalent (Bern et al. 2011; Messenger et al. 2015). If TcVI is new to Mexico, this strain could change the disease dynamics in a country that has enjoyed relatively

little chronic Chagas disease despite the abundance of infected *Triatoma* (Bern et al. 2011; Messenger et al. 2015).

Tcbat, considered to be a bat-exclusive lineage of *T. cruzi*, has not been reported north of Panama (Brenière et al. 2016). Tcbat was described from Brazilian bats showing unique patterns of ribosomal and spliced leader PCRs not clustering into any of the other six DTUs (Cavazzana et al. 2010; Ramírez et al. 2014; Lima et al. 2015). Tcbat has been reported in bats in from Brazil to Panama (Brenière et al. 2016), in a 5 year old girl living in northwestern Columbia (Ramírez et al. 2014), and even in mummies from Chile (Guhl et al. 2014). Tcbat is closely related to TcI and can be mistaken for TcI using standard molecular identification (Lima et al. 2015). The identification technique we used allows us to distinguish Tcbat from other DTUs by targeting a single molecular marker, the *TcSC5D* gene (Cosentino and Agüero 2012). While we only encountered one *T. longipennis* harboring Tcbat, 33 species of bats (7 families) inhabit the forests surrounding the field station (Ceballos and Miranda 2000). Given the high diversity of bat species in the region, it is not surprising to find Tcbat in our *Triatoma* sample. There is evidence of bat species *Glossophaga soricina*, *Mormoops megalophylla*, and *Pteronotus parnelli* infected with *T. cruzi* in Jalisco (Sánchez-Cordero et al, unpublished).

Triatoma at EBCh

We only encountered two species of Triatominae at EBCh, identified as *T. bolivari* and *T. longipennis* (Figure 1). Several other Triatominae that transmit *T. cruzi* have been reported in the state of Jalisco including *T. barberi, T. brailovskyi, T. dimidiata, T. phyllosoma, T. pallidipennis, and T. mazzotti* (Ramsey et al. 2015). *Triatoma* are known to have high genetic variability, which may be due to their continuous domestication process and fragmentation of habitats (Espinoza et al. 2013).

The *T. bolivari* were identified by physical characteristics and the *cyt b* gene sequence, which had a 91% match to a *T. bolivari* sequence collected at EBCh (Table 6) (Espinoza et al. 2013). Little work has been done on this *Triatoma* and few specimens have been collected. The initial report of the species, *T. bolivari*, was from a specimen collected in coastal Jalisco (Carcavallo et al. 1987). GeneBank only has 6 sequences reported to be from *T. bolivari*. The only study that has looked at their genetic diversity suggests that there is little diversity within this group, which is not consistent with the relatively low 91% identity for *cyt b* reported here (Espinoza et al. 2013).

Triatoma bolivari has been considered to be of low epidemiological importance (Ramsey et al. 2000). However, our study found 46% of *T. bolivari* infected with *T. cruzi* and most were collected near outdoor lights or inside EBCh buildings. We also identified human blood in a *T. bolivari* found inside the Library at EBCh. Light attraction by triatomines is believed to be a potential factor of infestation, which leads to higher chance of *T. cruzi* infection (Zeledón 1983; Rebollar-Téllez et al. 2009). *Triatoma bolivari* may be a strictly sylvatic species; adults attracted by human light sources are unable to colonize buildings. Martinez-Ibarra et al (2010) hypothesized that this species may be associated with birds rather than rodents. Our study found the predominant blood source in *T. bolivari* (3 of 5 triatomines with blood present) was the Mexican gray squirrel (*Sciurus aureogaster*). More research is needed to understand potential hosts and the role *T. bolivari* as a vector. However, it appears *T. bolivari* has disappeared from environmentally disturbed areas where it was previously reported and is now confined to coastal areas in western Mexico (Ramsey et al. 2000; Martinez-Ibarra et al. 2010).

Two reference samples of *T. longipennis*, male and female, were sent to UNAM for identification due to physical characteristics not matching reported *Triatoma*. The *COI* sequence

showed no significant homology to *T. longipennis* sequences found in GenBank. The highest homology was only 76% identity to *Triatoma vandae* (Table 6), a species from Brazil. We also sequenced the *cyt b* gene and found 87% sequence identity with deposited *T. longipennis* sequences, and 91% identity to *T. dimidiata.* Morphologically, the species we are calling *T. longipennis* is distinct from *T. dimidiata*. The Phyllosoma complex species, including *T. longipennis,* are reported to have large genetic variations making it difficult to distinguish species using molecular techniques. The relationships are further complicated by the ease of hybridization between species. We have called this species *T. longipennis* due to identification by UNAM despite the lack of sequence data to support this identification.

Blood meal sources

We targeted the *cyt b* and *COI* genes to identify the blood meal source when blood was observed in the gut contents of Triatominae. We were able to identify several blood meal sources from 11 samples, including *Homo sapiens, Odocoileus virginianus, Sciurus aureogaster, Canis familiaris, Gallus gallus,* and *Incilius* (*Bufo) marmoreus* (Table 7). All of these species are found in the study area (Myska 2011). Only two of the *Triatoma* with blood meal source data were negative for *T. cruzi*, both contained Mexican gray squirrel (*Sciurus aureogaster*) blood.

Not all of the *Triatoma* hosts identified through blood meal analysis are potential reservoirs of *T. cruzi.* Over 180 domestic and wild mammals, primarilay marsupials, xenarthras, bats, carnivores, lagomorphs, rodents, and primates, serve as hosts for *T. cruzi* (Carabarin-Lima et al. 2013). While many vertebrates can provide a blood meal for *Triatoma*, only mammals are known to support *T. cruzi*. The ability to survive in mammals is due in part to the parasite's expression of complement inhibitors to subvert innate immunity (Tambourgi et al. 1993; Norris 1998; Atayde et al. 2004; Cestari et al. 2008). Birds are known to be refractory to *T. cruzi*

infection owing to complement-mediated lysis of the parasite, as *T. cruzi's* complement inhibitors are mammalian-specific (Kierszenbaum et al. 1981). Therefore, while bird and amphibian blood was found in the *Triatoma* at EBCh, these animals are not contributing to *T. cruzi* transmission.

FTA paper

There are many advantages to collecting *Triatoma* gut contents and preserving the DNA on FTA paper. It protects DNA from degradation at room temperature and inactivates pathogens for safe handling. However, we found that storing the DNA of Triatominae on FTA paper is not optimal for PCR analysis. The amount of *T. cruzi* DNA retained on the FTA paper was considerably less than when DNA was extracted from a whole *Triatoma* and the distribution of DNA on the card was uneven. In addition, genomic DNA of *T. cruzi* is less abundant than kDNA, which made it difficult to amplify genomic sequences from *T. cruzi's 24Sα, 18S, TcSC5D* and *TcMK* genes. In the future, Triatominae samples should be preserved in 70% EtOH and whole specimens used for DNA extraction to maximize the DNA sample for analysis.

Potential risk of *T. cruzi* infection via wildlife

The high rates of infection of *Triatoma* leads to a concern of increased risk for people using wildlife as a protein source in the state of Jalisco. If an infected mammal is butchered in the field, there is a potential for transmission of the parasite from blood-borne exposure through open cuts or from blood spatter on mucus membranes. Little research has been published concerning *T. cruzi* transmission via hunting (Bern et al. 2011). Nevertheless, infection is a real risk, especially to those who skin and process wildlife meat themselves (Bern et al. 2011) and to those who feed on wildlife inhabiting lowland tropical deciduous forest in the region. Chagas

disease should be addressed to the community through public outreach efforts to avoid *T. cruzi* infection.

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